

# Natural habitats uncovered? – Genetic structure of known and newly found localities of the endangered bitterling *Pseudorhodeus tanago* (Cyprinidae)

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## Abstract

Overuse of natural resources by humans is a major threat to biodiversity. Overuse often involves species of economic or esthetic value, and fish are a typical example for a group that is exploited both for economic reasons (for human consumption) and for esthetic reasons (e.g. by aquarists). *Pseudorhodeus tanago* (Tanaka, 1909) (formerly known as *Tanakia tanago*) is a small colorful but legally protected (fishing, keeping and transfer are banned) bitterling fish distributed around Tokyo, Japan. Whereas it is critically endangered and more and more habitat loss has occurred, at least four stocks have been newly found during the last decade. To explore whether emergence of these newly found habitats is a consequence of incomplete survey, we genotyped mitochondrial cytochrome b sequence of *P. tanago* from 17 localities and an illegal home aquarium. Populations known by the past extensive survey (13 localities) showed geographically structured population genetic characteristics. Population-specific haplotypes were common indicating past divergence and bottleneck events. Four (north, {center + west}, south\_1, south\_2) or five

(north, center, west, south\_1, south\_2) geographic groups were detectable as for these known localities. On the other hand, newly found stocks were polymorphic and showed identical haplotypes from distant known localities. If we assume historical basis of distribution and genetic characteristics of these newly found stocks, it must be a series of unlikely geological events and haplotype sorting. We discuss potential issues posed by these questionable stocks.

### Keywords

Bottleneck, bucket biology, conservation genetics, fish dumping, home aquarium, phylogeography, poaching, satoyama, *Tanakia tanago*

## Introduction

Overuse of organisms by hunting or fishing for trade or esthetic purposes is one of the biggest threats to biodiversity. Controlling these activities is fundamental for ensuring the persistence of endangered organisms, particularly those that have traits attractive to humans. Alerts to poaching are thus necessary on those organisms legally protected. Not only tusks of elephants and rhinos but beautifully colored bodies of fish as well have attracted violators. The red list of Japanese brackish and freshwater fish (Ministry of the Environment, Japan 2015) acknowledges 39 species out of 168 of endangered and vulnerable categories have threats of overfishing in connection with home aquarium. Inversely, unauthorized fish release also threatens the integrity of natural fish population structures. A big source of invasion of alien freshwater fish is fish dumping from home aquaria (Lintermans 2004, Gertzen et al. 2008, Fuller et al. 2013, Ishikawa and Tachihara 2014). Poaching and dumping of endangered and protected fish, if any, disturb conservation programs and policy making and ultimately threat that species.

*Pseudorhodeus tanago* (Tanaka, 1909) (formerly known as *Tanakia tanago* transferred to the new genus, Chang et al. 2014) is a colorful small bitterling fish endemic to Japan with a limited geographic range around Tokyo. Habitats of *P. tanago* are in small water bodies such as ditches or ponds with spring water in hills (Nakamura 1969, Mochizuki 1997, Maita 2002, Ishinabe 2014). These habitats link with traditional agricultural landscape known as ‘satoyama’. Traditional farming activities have maintained ditches, ponds and vegetation around waters in farmlands in hills (Kobori and Primack 2003), and thus maintained habitats of *P. tanago*. However, because of heavily populated and highly developed areas around its habitats, *P. tanago* is critically endangered. Urbanization accompanying change of farming style gives rise to habitat loss (Mochizuki 1997, Ishinabe 2014). Agency for Cultural Affairs, and Ministry of the Environment, Japan have endorsed this bitterling as a legally protected species (Ministry of the Environment, Japan 2015). Fishing, keeping and transfer of *P. tanago* are banned. Since around 1970, conservation measures of *P. tanago* have been taken under local bases including extensive search for habitats, development of ex situ breeding techniques, protected area enclosure, mitigation of civil engineering activities (Tochigi Prefectural Fisheries Experimental Station 1973, Akiyama et al. 1994, Mochizuki 1997, Kubota et al. 2010, Ishinabe 2014). A national breeding program for the

protection of the species began in 1995, which included ex situ breeding and habitat restoration (Agency of Environment, Japan et al. 1995).

Records of 45 localities of *P. tanago* habitats were established as part of an extensive survey (Tanaka 1909, Nakamura 1969, Maita 2002, Ishinabe 2014, present report) (Fig. 1a). Some of the localities have been kept secret by authorities because of risks of poaching. By around 2000, most known habitats were lost, and further local extinction has occurred in these days in spite of conservation measures (Fig. 1b). Many populations have undergone habitat degradation to decline to ca. 1/100 in a few years, and in an extreme case the population is vanishing in the final remnant habitat of a short (< 100 m) stretch of a small (ca. 60 cm wide) stream (Mochizuki 1997).

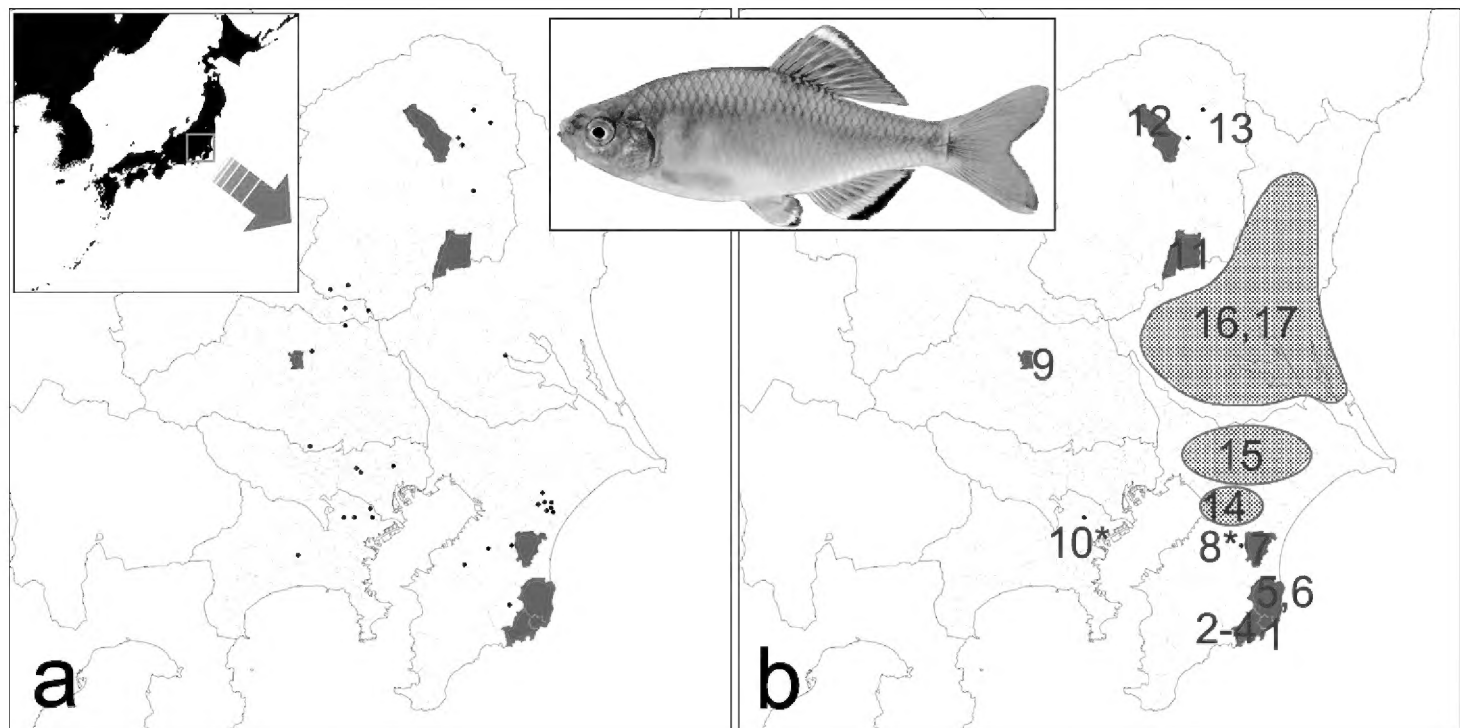
From the habitat characteristics of *P. tanago*, confined and scattered among headwaters of fine branching dales in agricultural landscape on hill terrains, we assume geographically structured population genetic characteristics of this species. Kubota et al. (2010) outlined geographic population structure of this species, but lack of localities from southern part of its geographic range and usage of questionable specimens obscured clear-cut geographic structure. Analysis of geographic population structure of this critically endangered fish with habitat loss based on specimens from reliable sources is necessary for setting up conservation programs (Waples and Gaggiotti 2006).

Whereas more and more local populations have been lost recently, *P. tanago* were newly found in a few localities in the last decade ('newly found' locality or stock hereafter) (Fig. 1b, hatched areas). Are these newly found stocks are simply because of incomplete survey of distribution? The aims of this study were to delineate geographical genetic structure of *P. tanago* and to identify symptom of disturbance, if any, in this structure by an analysis of genotypes of 80 individuals from 18 populations or stocks. Four of them are from newly found localities, one from an illegal home aquarium, and the others are from localities previously known by the past extensive survey ('known' localities or populations hereafter).

## Materials and methods

*Pseudorhodeus tanago* from 13 known localities collected from 1993 through 2013 (55 individuals), four newly found stocks collected from 2010 through 2014 (22 individuals), and three individuals seized from an illegal home aquarium were materials of this research (Table 1, Fig. 1b). Fishes from two out of the 13 known localities were of ex situ preserved stock extinct in the wild in early 1990s (#8) and late 1970s (#10). Habitat characteristics of the collecting localities were identical to descriptions in literatures (Nakamura 1969, Mochizuki 1997, Maita 2002) except for two newly found localities (#14, 17) where civil engineering activities including straight cut of channels with concrete enforcement have suffered their habitats.

Extraction of DNA was done from fin clips with QuickGene DNA Tissue kit on QuickGene-810 (Kurabo, Neyagawa, Japan). PCR primers were L14695 on the L-strand (AATTYTTGCTCRGACTCTAACC) and H15910 on the H-strand



**Figure 1.** Past (a) and present (b) geographic distribution of *Pseudorhodeus tanago*. Dots indicate records of presence of the bitterling. Light blue painted areas contain one to three localities from which specimens of the present report came. Exact places of these localities are kept secret by authorities to protect from poaching. Numbers on map b stand for localities of samples listed in Table 1. Asterisks indicate extinction in the wild and specimens came from ex situ preserved stocks. Habitats #14–17 in hatched areas (exact places are kept secret by authorities) are newly found. Hatched areas thus do not represent exact geographic ranges.

**Table 1.** Localities and specimens used in this study.

#	Locality	Collecting date, year	Number of individuals
1	Onjuku, Chiba	Dec. 19, 2012	4
2	Katsuura 1, Chiba	Jun. 14, 2012	8
3	Katsuura 2, Chiba	Jun. 14, 2012	4
4	Katsuura 3, Chiba	Aug. 22, 2012	4
5	Isumi A, Chiba	Jun. 21, 2012	4
6	Isumi B, Chiba	Jun. 26, 2012	8
7	Mobara, Chiba	Oct. 26, 2012	4
8	Nagara, Chiba*	Jan. 28, 2013	4
9	Namegawa, Saitama	2003	5
10	Small pond near Yokohama*	2003 year class	3
11	South-east Tochigi	2006	3
12	North Tochigi	1993-1994	1
13	Handa natural habitat conservation area, Tochigi	1993	3
14	Central Chiba**	May 16, 2010	3
15	North Chiba**	Jun. 3, 2010, Jul. 25, 2011	2
16	Ibaraki A**	Nov. 6, 2014	16
17	Ibaraki B**	2014	1
18	Seized from a home aquarium	2014	3

\* Preserved stock ex situ (extinct in the wild).

\*\* Newly found stocks.

(GATCTTCGGATTACAAGACCGAT) which worked for amplifying a 1219 bp mitochondrial DNA fragment encompassing the whole cytochrome b gene and flanking tRNA partial sequences. PCR reaction mixture of 12.5  $\mu$ L contained 1  $\mu$ L of template DNA, 0.96  $\mu$ L of dNTP mix (2.5 nmol each), 1.2  $\mu$ L of 10x ExTaq buffer, 0.06  $\mu$ L (0.3 U) of ExTaq (Takara, Shiga, Japan), 1  $\mu$ L of primers (5 pmol each), 7.28  $\mu$ L of Milli-Q grade water. PCR reaction was of touchdown profile (Don et al. 1991) in which annealing temperatures dropped from 59°C down to 53°C in the initial 7 cycles and was constant at 55°C in the remaining 28 cycles (35 cycles in total). The PCR reaction started with 3 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at the above touchdown annealing temperatures, 120 sec at 72°C with final extension at 72°C for 5 min. The PCR primers and two internal primers (L15438 [TTTTCCTACAAAGATCTATTAGG] and H15569 [CGTAAGATGGCGTAG-GCAAATAA] on the L- and H-strand, respectively) worked for overlapping double-stranded sequencing with BigDye terminator v.3.1 kit run on an ABI3730 sequencer (ABI, Foster City CA, USA). The complete mitochondrial cytochrome b sequences used in this research have DDBJ/GenBank entries of LC17598–LC170677.

Indices of nucleotide divergence were calculated with Arlequin v.3.5 (Excoffier and Lischer 2010). Geographic population structure was assessed with SAMOVA v.2.0 (Dupanloup et al. 2002). The net nucleotide divergence was calculated by  $\pi_{xy} - (\pi_x + \pi_y)/2$  where  $\pi_{xy}$  is average number of nucleotide differences between populations x and y, and  $\pi_x$  and  $\pi_y$  stand for this value between individuals within populations x and y. Parsimonious haplotype network was drawn with TCS v.1.2.1 (Clement et al. 2000).

## Results

### Haplotype grouping

Sequencing *Pseudorhodeus tanago* mitochondrial cytochrome b revealed 10 haplotypes (Table 2, Hap01–10). There were 19 variable sites, and 14 nucleotide substitutions observed between the most distant haplotypes (Hap01, 10) (Table 3). All the observed nucleotide substitutions were transitions, two of which were non-synonymous (positions 15100 and 15415). Most specimens from known localities (11/13) were monotypic, whereas newly found and seized stocks except for #17 with a single individual examined were polymorphic.

A haplotype network coincides with geographically structured population genetic characteristics of this species (Fig. 2). Regarding 13 known localities, haplotypes from each locality frequently were specific to that locality (9/13). Haplotypes placed on the upper-left side of the figure (Hap09, 10) were from northern part of the geographic range (Fig. 1b, #12, 13). Likewise, haplotypes arranged on the right side (Hap01–05) were all from the southern part (Fig. 1b, #1–6), and Hap08 on the lower-left side of the network was from the western part (Fig. 1b, #10) of the range. Placement of the other two haplotypes (Hap06, 07) was on the center of the network, and their geo-



**Table 2.** Haplotype composition of localities.

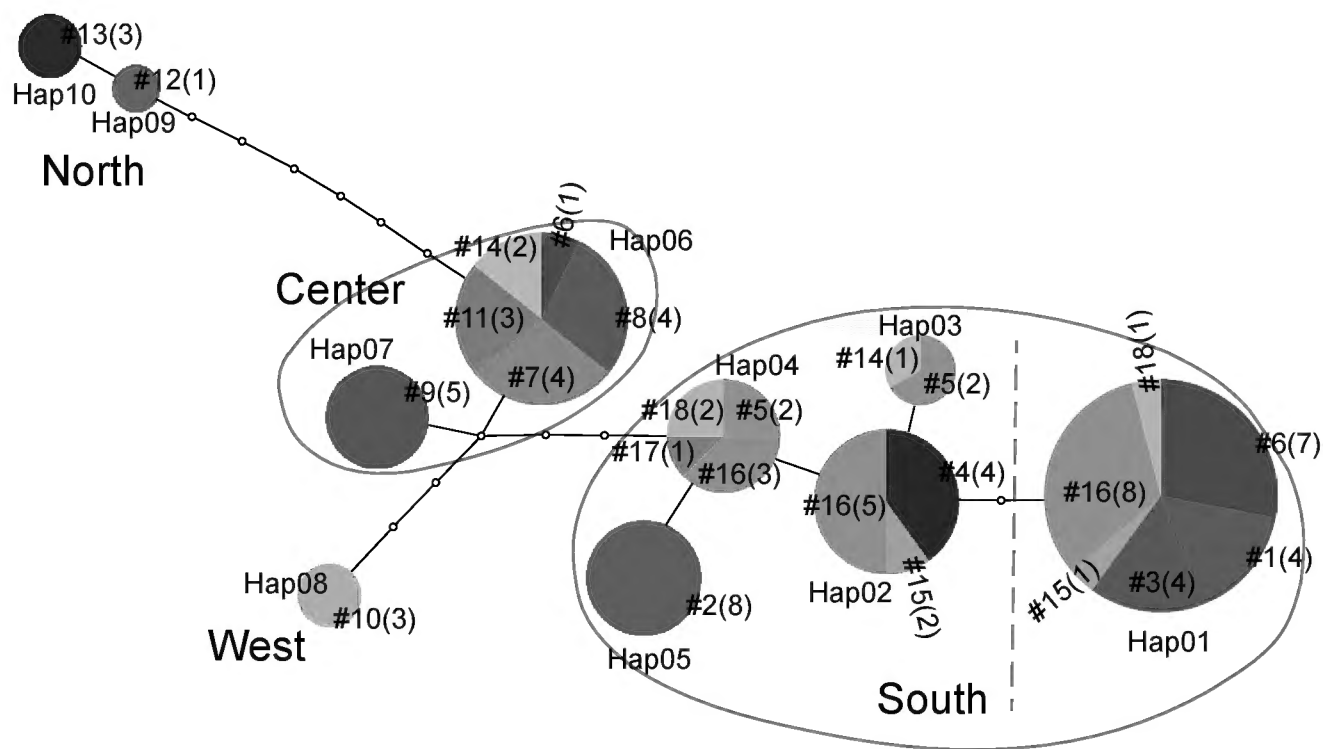
# \ Haplotype	Hap01	Hap02	Hap03	Hap04	Hap05	Hap06	Hap07	Hap08	Hap09	Hap10
1	4									
2					8					
3	4									
4		4								
5			2	2						
6	7					1				
7						4				
8*						4				
9							5			
10*								3		
11						3				
12									1	
13										3
14**			1			2				
15**	1	1								
16**	8	5		3						
17**				1						
18	1			2						

\* Preserved stock ex situ (extinct in the wild).  
\*\* Newly found stocks.

graphic placement was roughly the center of the range (Fig. 1b, #7–9) except for a single exception from locality #6 in the southern part (Table 2). This haplotype grouping was delineated by gaps with three or more missing haplotypes, whereas one or no gap lay between haplotypes within these groups. Haplotypes of the south group, however, contained distant types (four nucleotide differences at most), and there might be sub-grouping among them.

**Geographic grouping**

Geographic grouping of localities by AMOVA (SAMOVA) under assumptions of four or five geographic groups was similar to the haplotype grouping based on the haplotype network without geographic information (Table 4, Fig. 3). Values of sum of squares and coverage of variation among geographic groups were much higher than those values both among localities within geographic groups and within localities indicating clear geographic structures. The North haplotype group (Hap09, 10) appeared in a North geographic group that consisted of localities #12, 13. Regarding 13 known habitats, the SAMOVA grouping divided the South haplotype group into two geographic groups (south\_1 of localities #1, 3, 6 and south\_2 of #2, 4, 5). The former consisted predominantly of Hap01 which did not appear among specimens from the latter. The Center (#7–9, 11) and the West (#10) geographic groups under



**Figure 2.** Parsimonious haplotype network of mitochondrial DNA sequences of *Pseudorhodeus tanago* (Hap01–10). Each line connecting haplotypes indicates one nucleotide difference. Small open circles stand for missing haplotypes. Locality number (#) and number of individuals observed (parentheses) indicate haplotype sharing. Blue solid lines encircle haplotype groups (north, center, west and south). Blue broken line indicates sub-grouping within the south haplotype group exhibited by SAMOVA analysis (right, south\_1; left, south\_2).

**Table 3.** Haplotypes and variable sites of *Pseudorhodeus tanago* mitochondrial cytochrome b.

	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Nucleotide site *	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5
	4	4	5	5	5	5	8	9	9	9	9	0	1	2	3	4	4	4	5
	1	7	2	2	2	8	0	3	5	6	9	3	0	2	0	1	3	4	1
	2	2	0	6	9	0	7	7	5	7	4	0	0	8	1	5	2	7	3
Haplotype													**			**			
Hap01	T	G	A	C	G	G	G	C	G	G	G	T	A	A	T	G	C	A	G
Hap02	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	G	C	A	G
Hap03	T	G	A	C	G	G	A	C	G	G	G	C	A	G	T	G	C	A	G
Hap04	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	G	T	A	G
Hap05	T	G	A	C	G	G	A	C	G	G	G	C	A	A	T	A	T	A	G
Hap06	T	A	A	T	G	G	A	C	G	C	A	C	A	A	T	G	T	A	G
Hap07	T	A	A	T	G	G	A	C	G	G	A	C	A	A	C	G	T	A	G
Hap08	T	A	A	T	G	A	A	T	G	G	A	C	A	A	T	G	T	G	G
Hap09	C	A	G	T	A	G	A	C	A	A	A	C	G	A	T	G	T	A	A
Hap10	C	A	G	T	A	G	A	C	A	A	A	C	G	A	T	G	T	G	A

\* Positions corresponding with those of AP012526 (Miya et al. 2015).

\*\* Nucleotide sites with non-synonymous substitutions.

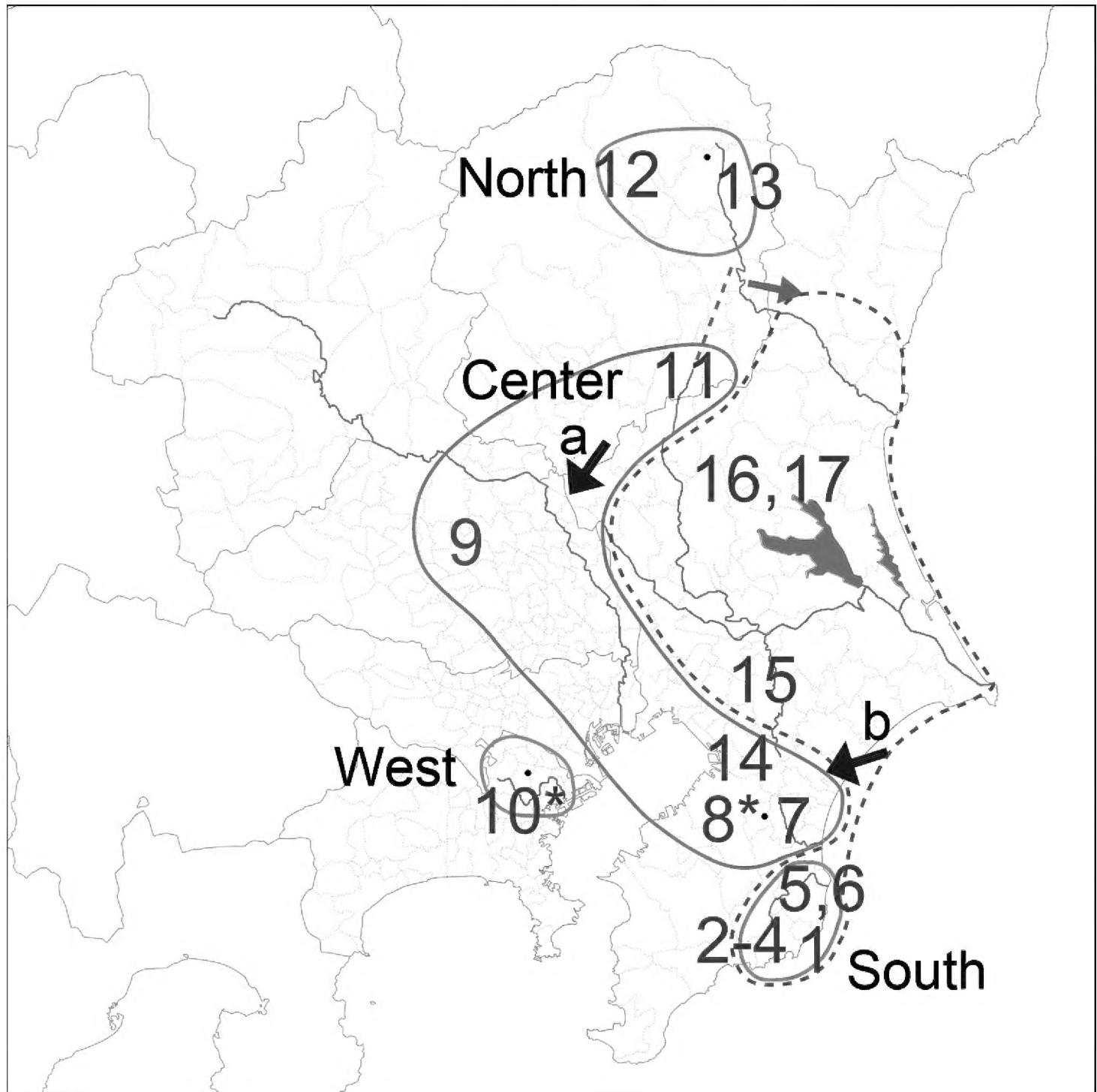
an assumption of five groups coincided with those of haplotype groups, but they were combined under a four groups assumption. Small but clear-cut nucleotide differences between localities within the south haplotype groups in spite of geographic proximity

**Table 4.** Contribution to variation in different patterns of population/stock grouping.

Grouping	Number of groups	Among groups				Among localities within groups				Within localities				Variance among groups
		Sum of squares	% of variation	Fct	P(Fct)	Sum of squares	% of variation	Fst	P(Fst)	Sum of squares	% of variation	Fsc	P(Fsc)	
Known localities only														
North + center + west + south_1 (#1 + #3 + #6) + south_2 (#2 + #4 + #5)	5	110,494	80,50	0,805	low*	15,562	13,35	0,939	low	8,125	6,15	0,685	low	2,532
North + {center + west} + south_1 (#1 + #3 + #6) + south_2 (#2 + #4 + #5)	4	101,475	74,43	0,744	low	24,582	19,52	0,940	low	8,125	6,04	0,764	low	2,382
All localities (known + newly found)														
North + {center + #14} + west + south_3 (#2) + south4 (others)	5	121,061	76,18	0,762	low	22,598	11,29	0,875	low	23,562	12,52	0,474	low	2,389
North + {center + #14} + west + south	4	105,356	72,76	0,728	low	38,302	16,08	0,888	low	23,562	11,16	0,590	low	2,560

\* P-value lower than 0.00001.





**Figure 3.** Geographic grouping of localities (encircled by gray solid lines) and river connectivity in 16th century (blue solid lines). Gray broken line encircles localities with the South geographic group including newly found localities (#15–17). Another newly found locality (#14) could be a member of the Center geographic group, but its haplotype composition is not typical among localities of the group. Arrows a and b denote historical overflow or stream capture terrains between river basins. Blue broken line indicates ancient river flow, and a blue arrow denotes change of the river flow at 500 ka.

(ca. 1–16 km, Table 5) divided the southern localities into two geographic groups, whereas moderate differences (0 to four base changes) among localities of the center and west geographic groups distant from each other (ca. 60–100 km except between #7 and #8) could be pooled.

Geographic grouping of known localities was largely coincident with river connectivity (Fig. 3). The North and the West geographic groups appeared each in a single river basin respectively. The South geographic group covered two river basins close to each other, one of which is kept secret by the authority (not shown on the map).

**Table 5.** Genetic ( $\pi_{xy}$ , below diagonal) and geographic (km, above diagonal) distance among collecting localities.

#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1		3.45	4.41	5.10	15.18	6.54	32.34	30.18	131.78	77.30	137.40	186.42	192.10	44.87	61.76	100.22	97.24
2	4.000		1.00	2.41	15.54	9.22	32.37	29.88	129.87	74.61	137.21	186.22	192.17	44.35	61.48	101.46	96.42
3	0.000	4.000		1.79	16.07	10.19	32.75	30.17	129.62	74.09	137.49	186.49	192.51	44.57	61.75	102.10	96.52
4	2.000	2.000	2.000		17.85	11.39	34.54	31.94	130.99	75.05	139.25	188.25	194.29	46.31	63.51	103.84	98.20
5	2.333	1.333	2.333	0.333		11.27	17.19	15.30	119.45	68.91	122.25	171.28	176.92	30.06	46.69	86.17	82.61
6	0.000	3.250	0.000	1.500	1.708		28.08	26.52	130.38	78.05	132.94	181.94	187.25	41.26	57.63	94.19	93.82
7	7.000	5.000	7.000	5.000	4.333	5.250		3.81	105.55	61.36	105.06	154.09	159.82	13.64	29.55	71.49	65.94
8	7.000	5.000	7.000	5.000	4.333	5.250	0.000		105.40	59.23	107.34	156.35	162.36	14.76	31.62	75.08	67.31
9	7.000	5.000	7.000	5.000	4.333	5.500	2.000	2.000		62.86	70.58	98.52	112.62	92.31	82.41	109.34	51.58
10*	9.000	7.000	9.000	7.000	6.333	7.500	4.000	4.000	4.000		103.40	146.69	140.01	52.65	55.93	106.07	59.01
11	7.000	5.000	7.000	5.000	4.333	5.250	0.000	0.000	2.000	4.000		49.02	56.15	93.02	75.74	61.16	45.70
12	13.000	11.000	13.000	11.000	10.333	11.375	7.000	7.000	8.000	10.000	7.000		15.79	141.99	124.74	103.09	92.74
13	14.000	12.000	14.000	12.000	11.333	12.375	8.000	8.000	9.000	9.000	8.000	1.000		148.38	130.99	103.10	101.6
14**	3.667	2.333	3.667	1.667	1.000	2.333	0.000	0.000	1.333	3.333	0.000	6.667	7.667		17.40	65.84	52.56
15**	0.000	2.000	0.000	0.000	0.333	-0.250	5.000	5.000	5.000	7.000	5.000	11.000	12.000	1.667		52.55	37.24
16**	3.000	1.000	3.000	1.000	0.333	2.250	4.000	4.000	4.000	6.000	4.000	10.000	11.000	1.333	1.000		58.96
17**	0.492	2.117	0.492	0.492	0.638	0.195	5.117	5.117	5.117	7.117	5.117	11.117	12.117	1.908	-0.508	1.117	

\* Preserved stock ex situ (extinct in the wild).

\*\* Newly found stocks.

Potential connection between these two basins through deltas is possible. The Center geographic group spanned a wider range on the periphery of a lowland plain which roughly corresponds with the area encircled by the gray solid line of this group plus coastal area on the east (Fig. 3). Localities of this geographic group presented in three river basins potentially connected with each other through flat low overflow terrain of 12 m high (Fig. 3, arrow a) or overflow plus switching dales (Kagose 1979) (flat hill top at 90m high and opposing headwaters with imbalanced slopes at 60 m high, arrow b).

On the other hand, placement of specimens from newly found localities onto the haplotype network obscured the geographic population genetic structure, though SAMOVA analysis apportioned these localities to either of the South or Center geographic groups according to their haplotype composition. Coverage of variation among geographic groups reduced from 74.43–80.5% to 72.76–76.18% (Table 4). Newly found localities except for #14 contained haplotypes of only the South group in spite of potential river connectivity with localities with the Center or the North geographic groups (Fig. 3). Locality #14 showed a mixed composition of both the Center and the South haplotype groups.

## Discussion

### Genetic architecture of known populations

The low sequence diversity of *Pseudorhodeus tanago* represented as monotypy in most known localities (11/13) conformed to their habitat characteristics (Table 2). Specific haplotypes from many of the known localities (9/13) indicated that populations of *P. tanago* rarely exchange with each other. Confinement in small isolated water bodies and subsequent bottleneck events might have brought about the present genetic structure of populations.

Isolation on a wider and geological scale is also responsible for divergence among geographic groups. The North geographic group contained haplotypes distant from others (Hap01 and Hap10), and ancestors of the North and other geographic groups thus diverged first. The central part of the entire geographic range of *P. tanago* encircled the flat plain that underwent repeated marine transgressions (Fig. 3). Marine transgression at > 400 ka (Sugai et al. 2013) and/or change of river flow at 500 ka (Fig. 3, blue arrow) (Koike et al. 1985, Kubota et al. 2010) might be responsible for the isolation of these geographic groups. Repeated marine transgressions afterward have facilitated further isolation among geographic groups.

### Implication of genetic characteristics of newly found stocks

Polymorphism and haplotype composition among newly found stocks, on the other hand, posed a question about the characteristics of genetic and geographic population

structure of *P. tanago*. If we assume historical basis of distribution and genetic characteristics of these newly found stocks, it must be a series of unlikely geological events and haplotype sorting.

Including these newly found stocks, *P. tanago* as a whole showed a bipartite population structure in which some localities show genetic variation while others rarely do so (Table 2). We should assume population admixture and rampant migration among these newly found stocks or one way migration from northwest part of the map including known populations (Fig. 1a), whereas known populations underwent isolation and bottleneck events. Marine transductions were, however, more frequent near the east coast since geological ancient until historic medieval times where newly found stocks inhabit (Kubo 2007, Sugai et al. 2013), and thus habitats of newly found stocks might be ephemeral prone to bottleneck. River connectivity implies that the hypothetical migration was from central or north geographic group (Fig. 3), but newly found localities (#15–17) consisted solely of south haplotype group. Admixture between populations around south group of known and newly found localities upon age marine regression is unlikely because of absence of continental shelf at the south-east coast (Sugai et al. 2013). Even if it was possible, river connection should involve localities of center geographic group (#7, 8) (Fig. 3), the main haplotype of which is absent from the newly found localities. Among newly found stocks, #14 consisted of haplotype of center group and was close to populations of center geographic group (#7, 8), but its natural distribution is questionable because of disturbance of the habitat by civil engineering. Straight cut channelization with concrete enforcement both on the banks and bottom has eliminated mud-sandy substrates for bitterlings' spawning host mussels.

There is no information such as confession or witness of violators about unauthorized releasing of *P. tanago* into the newly found localities. Genetic polymorphism of newly found localities may arrange stocks of these localities as conservation target of higher priority, if habitats are under risks of extinction. Recent decline of habitats indicates mitigation activities have not worked well (Mochizuki 1997, Ishinabe 2014) or just under trials (Tsunagawa et al. 2016). Then, ex situ breeding which works currently (Kubota et al. 2010, Ishinabe 2014) would be an interim choice. Under the assumption of natural distribution, stocks from newly found localities would be of higher priority for ex situ preservation.

Natural distribution in these newly found localities is, however, questionable because of unlikely association between haplotype/population relationships and river connectivity. Because bitterling fishes have attractive coloration to humans, there have been reports on unauthorized intentional releasing activities possibly implicated in home aquaria (Miyake et al. 2011, Kitazima et al. 2015, Saitoh et al. 2016). Under an assumption of unauthorized releasing or dumping of *P. tanago*, we confront difficult issues. Genetic polymorphism of the newly found and seized stocks (Table 2) implied a number of poaching activities. Because violators do not assess stock size of their target fish in the field, poaching would sometimes be devastating. Unauthorized releasing or dumping, if any, also poses problems. Unauthorized releasing of *P. tanago* is illegal, because unauthorized transfer of this legally protected fish is prohibited. Released *P.*

*tanago* individuals themselves are, however, legal target of conservation (Ministry of the Environment, Japan 2015). Presence of questionable stocks thus disturbs prioritization of conservation targets under limited funds and human resources. Releasing or dumping onto habitats of natural populations is a direct threat to the integrity of natural properties of *P. tanago*. Our report has cautionary implication to potential violators.

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